

## Accumulation of constitutive diterpenoids in the rhytidome and secondary phloem of the branch bark of *Larix gmelinii* var. *japonica*

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**Abstract** The quantitative compositions of the major constitutive diterpenoids in the rhytidome and secondary phloem of the branch bark of Kuril larch (*Larix gmelinii* var. *japonica*) were investigated. The eight major diterpenoids were isolated from a diethyl ether extract of the branch bark of *L. gmelinii* var. *japonica* and identified as 13-epimanol (1), larixol (2), larixyl acetate (3), 13-epitriolacetate (4), abietic acid (5), neoabietic acid (6), dehydroabietic acid (7), and isopimaric acid (8). The amount of each diterpenoid was subsequently quantified in both the rhytidome and secondary phloem. All of the diterpenoids were present in both bark tissues, but the amounts were significantly higher in the rhytidome than in the secondary phloem. Developed fusiform resin cavities containing oleoresins were commonly observed in dead secondary phloem captured into the rhytidome of a bark transverse section. The accumulation and distribution of these constitutive diterpenoids in the bark tissues can probably be attributed to terpenoid biosynthesis in the living secondary phloem and the rhytidome formation process. From the viewpoint of constitutive chemical defense in conifers, it is suggested that the tree body may be more effectively defended against natural enemies by the higher amount of diterpenoid amount in the outermost and dead bark tissue, the rhytidome, where the potentially

poisonous and easily oxidizable diterpenoids can be safely and stably maintained.

**Keywords** Constitutive terpenoids · Rhytidome · Secondary phloem · *Larix gmelinii* var. *japonica* · Plant chemical defense

### Introduction

The primary function of tree bark is to physically and chemically protect the tree body from weather and natural enemies [1, 2]. Constitutive defense by conifer bark against a great number of enemies, such as phytopathogens, herbivorous insects, and mammals, results from a combination of toxic chemicals, anatomical structures, and the location of the chemicals [3–5]. Healthy conifers constitutively produce and store oleoresins, whose major components are terpenoids that protect the trees by preventing outbreaks of pests [6–9]. Hence, constitutive terpenoids in conifer bark provide a very important front-line defense against enemies [5, 10].

Kuril larch (*Larix gmelinii* var. *japonica*) was introduced to Hokkaido Island, northern Japan, from Sakhalin and the southern Kuril Islands. *L. gmelinii* var. *japonica* is one of the important parent tree species for forest tree breeding on Hokkaido Island because it is superior to other introduced *Larix* species, such as Japanese larch (*L. kaempferi*), in its trait of resistance to bark consumption under snow by the native gray red-backed vole (*Myodes rufocanus bedfordiae*; formerly *Clethrionomys rufocanus bedfordiae*) [11–13]. The resistance of *L. gmelinii* var. *japonica* to the vole is considered to be a chemical-based defense that results from constituents in its bark. Previous studies have shown that the resistance can be attributed to resin cyst number/size, essential oil and/or crude fat

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contents in the bark [14, 15], and essential oil and/or crude resin (determined from diethyl ether extracts) contents in the bark [11, 16]. The degree of resistance to voles among the larches significantly correlated with the contents of the diethyl ether extracts of the bark [17]. Some diterpenoids in the petroleum ether extract from the branch bark of *L. gmelinii* var. *japonica* could be associated with the resistance to the vole [18]. Our previous report [19] also showed that the chemical composition of the branch bark tissue of *L. gmelinii* var. *japonica* was an effective index for discrimination of hybrid larch  $F_1$  (*L. gmelinii* var. *japonica*  $\times$  *L. kaempferi*) seedlings with superior growth rate and chemical defense, e.g., resistance to voles.

Mature bark basically consists of two types of bark tissues, rhytidome and secondary phloem, which are also referred to as the outer and inner bark, respectively [20]. The secondary phloem is living tissue, which is differentiated from the vascular cambium [21]. It biosynthesizes a large number of primary and secondary metabolites from assimilation products of photosynthesis and also transports and stores these metabolites [22]. In contrast, the rhytidome is dead tissue, which originally develops from the secondary phloem [21]. It physically protects the tree body [2]. Conifers, especially Pinaceae trees, biosynthesize and store many constitutive terpenoids in the living secondary phloem [10, 23]. However, little is known about the quantitative composition and distribution of terpenoids in the rhytidome of conifers. Understanding the details of bark chemical defense is important for forest tree breeding, and the role of the chemical defense of the rhytidome remains unclear.

We investigated the accumulation of the major constitutive diterpenoids in the rhytidome of the branch bark of *L. gmelinii* var. *japonica* and compared the contents with those in the secondary phloem to understand how defensive compound placement and anatomical structures in the conifer bark are integrated into constitutive defense. For this purpose, we isolated, purified, and identified diterpenoids from a diethyl ether extract of the branch bark of *L. gmelinii* var. *japonica*. Next, the diterpenoids were used as authentic compounds for their subsequent quantification in both bark tissues. In this report, we also discussed diterpenoid accumulation and anatomical formation of bark tissues for constitutive chemical defense in *L. gmelinii* var. *japonica*.

## Materials and methods

### Plant materials

Samples for diterpenoid analysis were collected from a 30-year-old *L. gmelinii* var. *japonica* tree at the Doto station of the Forestry Research Institute, Hokkaido Research

Organization, Shintoku town (200 m a.s.l., 43°04'N, 142°50'E), central Hokkaido Island. In September 2000, the branches were collected from the north side of 12 tree stems at an approximate height of 8 m using a handsaw with a long handle. All branch samples had developed rhytidome, and their age and end diameters were within 10–15 years and 3–5 cm, respectively. They were immediately cooled and placed in a laboratory freezer at  $-20^{\circ}\text{C}$  until chemical analyses were performed.

### General equipment

$^1\text{H}$  and  $^{13}\text{C}$  nuclear magnetic resonance (NMR) spectra were obtained using a JNM-AL400 spectrometer (JEOL, Tokyo, Japan) with standard pulse sequences operating at 400 and 100 MHz, respectively.  $\text{CDCl}_3$  (Sigma-Aldrich, St. Louis, MO, USA) was used as a solvent, and tetramethylsilane (TMS; Wako Pure Chemical Industries, Osaka, Japan) was used as an internal standard. Electron and chemical impact mass spectrometry (EIMS and CIMS) spectra were recorded on a JMS-600H spectrometer (JEOL), and the former was performed at an ionizing energy of 70 eV, while the latter was performed with ammonia or isobutane as the reagent gas at 250 eV. Infrared (IR) spectra were determined using a FT/IR-8900 spectrometer (Jasco, Tokyo, Japan).

Silica gel column chromatography (SiGCC) was performed on silica gel 60 (0.063–0.200 mm, Merck, Darmstadt, Germany) packed in a glass column (700 mm  $\times$  60 mm i.d.). Preparative high-performance liquid chromatography (HPLC) with gradient elution was performed using two LC-10A pumps, an SPD-10A ultraviolet (UV) detector (wavelength: 245 nm), and an FRC-10A fraction collector (all these equipment were from Shimadzu, Kyoto, Japan), and a Mightysil RP-18GP octadecylsilane (ODS) column (column size, 250 mm  $\times$  20 mm i.d.; particle size of the column substrate, 5  $\mu\text{m}$ ; Kanto Chemical, Tokyo, Japan).

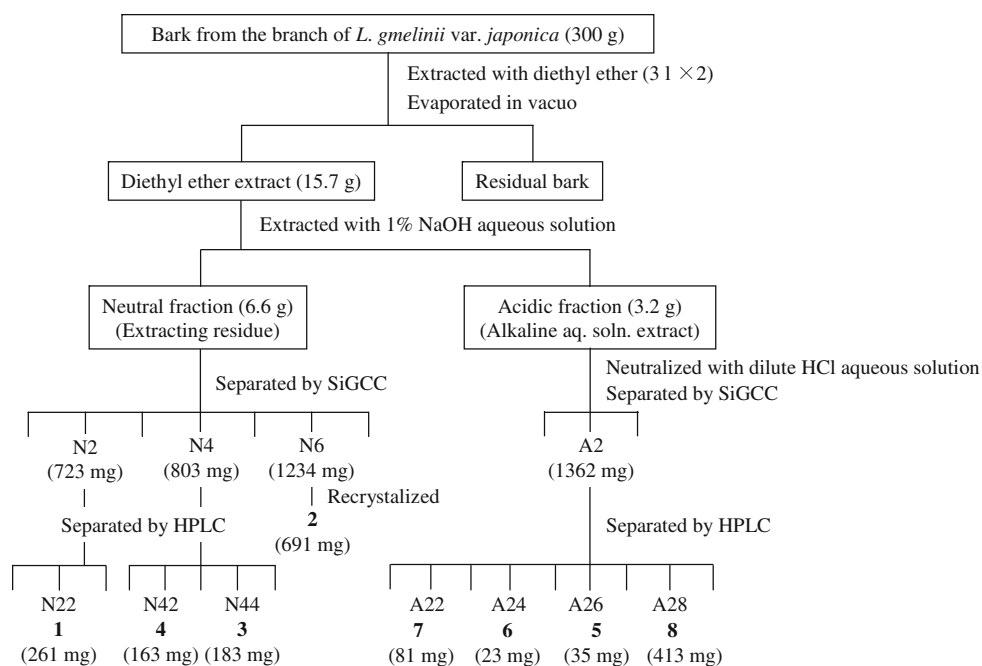
Gas chromatography (GC) was performed using a GC-14A gas liquid chromatograph equipped with a hydrogen flame ionization detector (FID) (Shimadzu), and a DB-1 fused silica capillary column (30 m  $\times$  0.32 mm i.d., 0.25  $\mu\text{m}$  film thickness; Agilent Technologies, Santa Clara, CA, USA). The column was heated from 170 to 280  $^{\circ}\text{C}$  at 5  $^{\circ}\text{C}/\text{min}$ . The injection and detection port temperatures were maintained at 280 and 290  $^{\circ}\text{C}$ , respectively, and helium was used as the carrier gas.

Optical microscopy and digital imaging were performed using a SMZ-U stereoscopic zoom microscope equipped with an E4500 digital camera (both from Nikon, Tokyo, Japan).

### Extraction and isolation of diterpenoids from the bark

The schema for separating the diterpenoids from the branch bark of *L. gmelinii* var. *japonica* is shown in Fig. 1. For the

**Fig. 1** Schema for separation of the diterpenoids from the branch bark of *Larix gmelinii* var. *japonica*. Compounds **1–8**: see Fig. 2



branches sampled from the individual trees, the bark near the stem side was peeled off using a steel scraper, crushed in a homogenizer with liquid nitrogen, and then lyophilized for 24 h. The freeze-dried bark meal (300 g) was extracted with diethyl ether (3 l  $\times$  2) for 2 days at room temperature. Evaporation of the extract in vacuo under a stream of nitrogen yielded a dark yellow viscid residue (15.7 g).

The extract was separated into neutral (6.6 g) and acidic (3.2 g) fractions according to solubility in 1 % NaOH aqueous solution, and these two fractions were each further separated by SiGCC and preparative HPLC using the ODS column (Fig. 1). The neutral fraction was separated into three fractions, designated N2 (723 mg), N4 (803 mg), and N6 (1234 mg), by preliminary SiGCC (silica gel, 900 g) and eluted with benzene (C<sub>6</sub>H<sub>6</sub>)–ethyl acetate (EtOAc) (8:2 v/v). Fraction N22 was purified from fraction N2 by preparative HPLC [eluent, acetonitrile (MeCN)–H<sub>2</sub>O (7:3 v/v); flow rate, 9.5 ml/min]. Fraction N4 was separated into fractions N42 and N44 using the previously described HPLC procedure. The acidic fraction was neutralized with dilute aqueous hydrochloric acid solution, preliminarily chromatographed on silica gel, and then eluted with C<sub>6</sub>H<sub>6</sub>–EtOAc (8:2 v/v) containing 0.1 % formic acid to obtain fraction A2 (1362 mg). The resulting fraction A2 was further separated into fractions A22, A24, A26, and A28 by preparative HPLC with gradient elution [eluent, MeCN–H<sub>2</sub>O (7:3–85:15 v/v); flow rate, 9.5 ml/min].

#### *13-Epimanool* [*labda-8* (17), *14-dien-13S-ol*] (**1**)

Compound **1** (261 mg) was obtained as an amorphous substance from fraction N22. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):

$\delta$  0.68 (s, 3H, 20-Me), 0.80 (s, 3H, 19-Me), 0.87 (s, 3H, 18-Me), 1.27 (s, 3H, 16-Me), 4.51 (s, 1H, 17-H), 4.82 (s, 1H, 17-H), 5.05 (dd, 1H,  $J$  = 1.2 and 10.8 Hz, 15-H), 5.20 (dd, 1H,  $J$  = 1.2 and 17.6 Hz, 15-H), 5.91 (dd, 1H,  $J$  = 10.8 and 17.2 Hz, 14-H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  14.4 (20-Me), 17.7 (11-CH<sub>2</sub>), 19.4 (2-CH<sub>2</sub>), 21.7 (19-Me), 24.4 (6-CH<sub>2</sub>), 27.6 (16-Me), 33.5 (4-C), 36.1 (18-Me), 38.3 (7-CH<sub>2</sub>), 39.0 (1-CH<sub>2</sub>), 39.8 (10-C), 41.3 (12-CH<sub>2</sub>), 42.1 (3-CH<sub>2</sub>), 55.5 (5-CH), 57.2 (9-CH), 73.4 (13-C–O), 106.2 (17-CH<sub>2</sub>), 111.2 (15-CH<sub>2</sub>), 144.9 (14-CH), 148.3 (8-C). EIMS (70 eV):  $m/z$  (%) 272 (M<sup>+</sup>–H<sub>2</sub>O, 69), 257 (91), 137 (100). CIMS (250 eV, ammonia):  $m/z$  (%) 308 (M<sup>+</sup>+NH<sub>4</sub>, 100), 290 (M<sup>+</sup>, 79), 273 (96). IR  $\nu_{\max}$  (CHCl<sub>3</sub>) cm<sup>–1</sup>: 3418, 3081, 2931, 1645, 1459, 918, 889.

#### *Larixol* [*labda-8* (17), *14-dien-6 $\alpha$* , *13S-diol*] (**2**)

Compound **2** (691 mg) was obtained as colorless microcrystals from fraction N6 by recrystallization with benzene. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  0.69 (s, 3H, 20-Me), 1.01 (s, 3H, 19-Me), 1.12 (s, 3H, 18-Me), 1.27 (s, 3H, 16-Me), 4.60 (s, 1H, 17-H), 4.89 (s, 1H, 17-H), 5.06 (dd, 1H,  $J$  = 1.2 and 10.8 Hz, 15-H), 5.20 (dd, 1H,  $J$  = 1.2 and 17.6 Hz, 15-H), 5.91 (dd, 1H,  $J$  = 10.6 and 17.4 Hz, 14-H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  16.0 (20-Me), 18.0 (11-CH<sub>2</sub>), 18.4 (2-CH<sub>2</sub>), 22.3 (19-Me), 27.7 (16-Me), 33.5 (4-C), 36.1 (18-Me), 38.3 (7-CH<sub>2</sub>), 39.0 (1-CH<sub>2</sub>), 41.3 (10-C), 43.7 (12-CH<sub>2</sub>), 49.1 (3-CH<sub>2</sub>), 56.4 (5-CH), 60.4 (9-CH), 71.5 (6-C–O), 73.3 (13-C–O), 108.2 (17-CH<sub>2</sub>), 111.3 (15-CH<sub>2</sub>), 144.8 (14-CH), 145.2 (8-C). EIMS (70 eV):  $m/z$  (%) 288 (M<sup>+</sup>–H<sub>2</sub>O, 35), 273 (27), 153 (46), 109 (83), 69 (100); CIMS (250 eV, ammonia):  $m/z$  (%)

324 ( $M^+ + NH_4$ , 100), 306 ( $M^+$ , 12), 296 (13); IR  $\nu_{max}$  (KBr)  $cm^{-1}$ : 3450–3350, 3085, 2929, 1648, 922, 877.

*Larixyl acetate [6 $\alpha$ -acetoxy-labda-8 (17), 14-dien-13S-ol] (3)*

Compound **3** (183 mg) was obtained as an amorphous substance from fraction N44.  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  0.74 (s, 3H, 20-Me), 0.87 (s, 3H, 19-Me), 1.01 (s, 3H, 18-Me), 1.26 (s, 3H, 16-Me), 2.05 (s, 3H, OAc), 2.68 (dd, 1H,  $J = 5.2$  and 12.4 Hz, 7-H), 4.64 (s, 1H, 17-H), 4.93 (s, 1H, 17-H), 5.06 (dd, 1H,  $J = 1.2$  and 10.8 Hz, 15-H), 5.20 (dd, 1H,  $J = 1.2$  and 17.2 Hz, 15-H), 5.91 (dd, 1H,  $J = 10.8$  and 17.2 Hz, 14-H).  $^{13}C$  NMR (100 MHz,  $CDCl_3$ ):  $\delta$  15.9 (20-Me), 18.0 (11- $CH_2$ ), 18.4 (2- $CH_2$ ), 21.9 (22-Me), 22.4 (19-Me), 27.7 (16-Me), 33.4 (4-C), 36.1 (18-Me), 39.0 (7- $CH_2$ ), 39.8 (1- $CH_2$ ), 41.2 (10-C), 43.4 (12- $CH_2$ ), 44.1 (3- $CH_2$ ), 56.2 (5-CH), 57.4 (9-CH), 73.1 (6-C-O), 73.3 (13-C-O), 109.2 (17- $CH_2$ ), 111.4 (15- $CH_2$ ), 143.9 (14-CH), 144.8 (8-C), 169.7 (21-COMe). EIMS (70 eV):  $m/z$  (%) 288 ( $M^+ - AcOH$ , 24), 270 (75), 255 (100). CIMS (250 eV, ammonia):  $m/z$  (%) 366 ( $M^+ + NH_4$ , 100), 348 ( $M^+$ , 15); IR  $\nu_{max}$  ( $CHCl_3$ )  $cm^{-1}$ : 3461, 3086, 2927, 1737, 1445, 1378, 1243, 1024, 919.

*13-Epitorulosyl acetate [19-acetoxy-labda-8 (17), 14-dien-13S-ol] (4)*

Compound **4** (163 mg) was obtained as an amorphous substance from fraction N42.  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  0.68 (s, 3H, 20-Me), 1.05 (s, 3H, 18-Me), 1.27 (s, 3H, 16-Me), 2.03 (s, 3H, OAc), 3.85 (d, 1H,  $J = 11.2$  Hz, 19-H), 4.22 (d, 1H,  $J = 11.2$  Hz, 19-H), 4.55 (s, 1H, 17-H), 4.82 (s, 1H, 17-H), 5.05 (dd, 1H,  $J = 1.2$  and 10.8 Hz, 15-H), 5.20 (dd, 1H,  $J = 1.4$  and 17.4 Hz, 15-H), 5.91 (dd, 1H,  $J = 10.8$  and 17.2 Hz, 14-H).  $^{13}C$  NMR (100 MHz,  $CDCl_3$ ):  $\delta$  15.2 (20-Me), 17.8 (11- $CH_2$ ), 18.9 (2- $CH_2$ ), 20.9 (22-Me), 24.4 (6-CH), 27.5 (18-Me), 27.6 (16-Me), 36.2 (3- $CH_2$ ), 37.2 (4-C), 38.4 (7- $CH_2$ ), 38.8 (1- $CH_2$ ), 39.6 (10-C), 41.2 (12- $CH_2$ ), 56.0 (5-CH), 57.2 (9-CH), 66.6 (19- $CH_2$ ), 73.3 (13-C-O), 106.7 (17- $CH_2$ ), 111.3 (15- $CH_2$ ), 144.9 (14-CH), 147.5 (8-C), 170.9 (21-COMe). EIMS (70 eV):  $m/z$  (%) 330 ( $M^+ - H_2O$ , 35), 315 (25), 288 (15), 270 (36), 257 (78), 135 (100). CIMS (250 eV, ammonia):  $m/z$  (%) 366 ( $M^+ + NH_4$ , 100), 348 ( $M^+$ , 85), 331 (43). IR  $\nu_{max}$  ( $CHCl_3$ )  $cm^{-1}$ : 3466, 3082, 2930, 1740, 1243.

*Abietic acid [abieta-7, 13-dien-18-oic acid] (5)*

Compound **5** (35 mg) was obtained as colorless microcrystals from fraction A26.  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  0.81 (s, 3H, 20-Me), 0.95 (s, 3H, 19-Me), 1.03 (d, 3H,

$J = 6.9$  Hz, 16-Me), 1.24 (d, 3H,  $J = 6.9$  Hz, 17-Me), 2.87 (sept, 1H,  $J = 6.9$  and 6.9 Hz, 15-H), 5.37 (m, 1H, 7-H), 5.77 (d, 1H,  $J = 2.4$  Hz, 14-H).  $^{13}C$  NMR (100 MHz,  $CDCl_3$ ):  $\delta$  14.0 (20-Me), 16.7 (19-Me), 18.0 (2- $CH_2$ ), 20.8 (17-Me), 21.4 (16-Me), 22.4 (11- $CH_2$ ), 25.6 (6- $CH_2$ ), 27.4 (12- $CH_2$ ), 34.4 (10-C), 34.8 (15-CH), 37.1 (3- $CH_2$ ), 38.2 (1- $CH_2$ ), 44.9 (5-CH), 46.2 (4-C), 50.8 (9-CH), 120.2 (7-CH), 122.1 (14-CH), 135.2 (8-C), 144.9 (13-C), 184.2 (18-COOH). EIMS (70 eV):  $m/z$  (%) 302 ( $M^+$ , 100), 287 (49), 259 (76), 270 (36), 239 (47), 136 (68). CIMS (250 eV, isobutane):  $m/z$  (%) 303 ( $M^+ + H$ , 100), 257 (20). IR  $\nu_{max}$  (KBr)  $cm^{-1}$ : 3413, 2935, 1460, 1386, 1278.

*Neoabietic acid [abieta-8(14), 13(15)-dien-18-oic acid] (6)*

Compound **6** (23 mg) was obtained as colorless microcrystals from fraction A24.  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  0.79 (s, 3H, 20-Me), 1.20 (s, 3H, 19-Me), 1.70 (d, 3H,  $J = 6.9$  Hz, 16-Me), 1.73 (d, 3H,  $J = 6.9$  Hz, 17-Me), 6.20 (d, 1H,  $J = 2.4$  Hz, 14-H).  $^{13}C$  NMR (100 MHz,  $CDCl_3$ ):  $\delta$  15.3 (20-Me), 16.8 (19-Me), 18.1 (2- $CH_2$ ), 19.7 (17-Me), 20.4 (16-Me), 22.3 (11- $CH_2$ ), 24.9 (6- $CH_2$ ), 25.8 (12- $CH_2$ ), 35.5 (7- $CH_2$ ), 37.1 (3- $CH_2$ ), 37.7 (10-C), 38.5 (1- $CH_2$ ), 47.3 (4-C), 48.6 (5-CH), 51.5 (9-CH), 122.2 (14-CH), 123.6 (15-C), 128.2 (13-C), 138.4 (8-C), 185.1 (18-COOH). EIMS (70 eV):  $m/z$  (%) 302 ( $M^+$ , 48), 148 (24), 135 (100). CIMS (250 eV, isobutane):  $m/z$  (%) 303 ( $M^+ + H$ , 100), 257 (36). IR  $\nu_{max}$  (KBr)  $cm^{-1}$ : 3413, 2935, 1694, 1456, 1281, 821.

*Dehydroabietic acid [abieta-8, 11, 13-trien-18-oic acid] (7)*

Compound **7** (81 mg) was obtained as colorless microcrystals from fraction A22.  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  1.21 (s, 3H, 20-Me), 1.26 (s, 3H, 19-Me), 1.23 (d, 3H,  $J = 6.9$  Hz, 16-Me), 1.23 (d, 3H,  $J = 6.9$  Hz, 17-Me), 2.24 (dd, 1H,  $J = 12.4$  and 2.4 Hz, 5-H), 2.88 (sept, 1H,  $J = 6.9$  and 6.9 Hz, 15-H), 6.88 (brs, 1H, 14-H), 7.16 (d, 1H,  $J = 2.4$  Hz, 11-H).  $^{13}C$  NMR (100 MHz,  $CDCl_3$ ):  $\delta$  16.2 (19-Me), 18.5 (2- $CH_2$ ), 23.9 (16-Me), 23.9 (17-Me), 25.1 (20-Me), 29.9 (7- $CH_2$ ), 33.4 (15-CH), 36.6 (3- $CH_2$ ), 36.8 (10-C), 37.8 (1- $CH_2$ ), 21.7 (6- $CH_2$ ), 44.5 (5-CH), 47.4 (4-C), 123.6 (12-CH), 123.8 (11-CH), 126.6 (14-CH), 134.3 (8-C), 145.4 (13-C), 146.4 (9-C), 184.0 (18-COOH). EIMS (70 eV):  $m/z$  (%) 300 ( $M^+$ , 31), 285 (100), 239 (72), 270 (36), 197 (28). CIMS (250 eV, isobutane):  $m/z$  (%) 301 ( $M^+ + H$ , 100), 255 (64). IR  $\nu_{max}$  (KBr)  $cm^{-1}$ : 3413, 2958, 1694, 1498, 1459, 1387, 1278.

*Isopimaric acid [13-epi-pimara-7, 15-dien-18-oic acid] (8)*

Compound **8** (413 mg) was obtained as colorless microcrystals from fraction A28.  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  0.86 (s, 3H, 20-Me), 0.91 (s, 3H, 19-Me), 1.26 (s, 3H,

17-Me), 4.87 (dd, 1H,  $J = 10.8$  and  $1.2$  Hz, 16-H), 4.93 (dd, 1H,  $J = 17.6$  and  $1.6$  Hz, 16-H), 5.80 (dd, 1H,  $J = 17.6$  and  $10.8$  Hz, 15-H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  15.3 (20-Me), 17.1 (19-Me), 17.9 (2- $\text{CH}_2$ ), 20.0 (11- $\text{CH}_2$ ), 21.5 (6- $\text{CH}_2$ ), 25.1 (17-Me), 34.9 (12- $\text{CH}_2$ ), 36.0 (3- $\text{CH}_2$ ), 36.7 (10-C), 36.9 (13-C), 38.7 (1- $\text{CH}_2$ ), 44.9 (5-CH), 46.0 (14- $\text{CH}_2$ ), 44.9 (4-C), 51.9 (9-CH), 109.1 (16- $\text{CH}_2$ ), 120.7 (7-CH), 135.3 (8-C), 149.9 (15-CH), 184.1 (18-COOH). EIMS (70 eV):  $m/z$  (%) 302 ( $\text{M}^+$ , 100), 287 (66), 257 (45), 241 (73), 187 (56). CIMS (250 eV, isobutane):  $m/z$  (%) 303 ( $\text{M}^+ + \text{H}$ , 100), 257 (32). IR  $\nu_{\text{max}}$  (KBr)  $\text{cm}^{-1}$ : 3412, 2936, 1637, 1458, 1348, 1280, 999, 910.

#### Quantitative analysis of the major constitutive diterpenoids in the rhytidome and secondary phloem

For each of the respective branch samples from the 12 individual trees, the bark from the nearby stem side was exactly separated at the border of the last formed periderm into the rhytidome and secondary phloem using a scalpel. Samples of the rhytidome and secondary phloem were separately homogenized in a grinder with liquid nitrogen. The chemical analysis methods used for the rhytidome and secondary phloem were modified from those described in a previous report [24]. A 200-mg sample of the bark tissue meal was extracted with 4 ml of petroleum ether–diethyl ether (1:1 v/v) containing 200  $\mu\text{g}/\text{ml}$  heptadecanoic acid (Sigma-Aldrich) as an internal standard in a screw-cap test tube during ultrasonication in a water bath for 1 h under cooling at approximately  $10^\circ\text{C}$ . The sample was centrifuged, and two 1-ml supernatant samples were evaporated to dryness under a stream of nitrogen. The content of the petroleum ether–diethyl ether extract in one of the dried samples was quantified. The other dried extract was methylated with ethereal diazomethane to derive acidic diterpenoids as their methyl esters. The major diterpenoids in the methylated samples were analyzed by GC. The eight isolated, purified, and identified diterpenoids were used as standard compounds. The peaks in the chromatograms of the diterpenoids in the samples were identified by comparing their retention times with those of the standard diterpenoids, and the diterpenoid contents were quantified using calibration curves. The contents of the derived acidic diterpenoid methyl esters were recalculated on the basis of their original corresponding acidic diterpenoids.

#### Statistical analysis

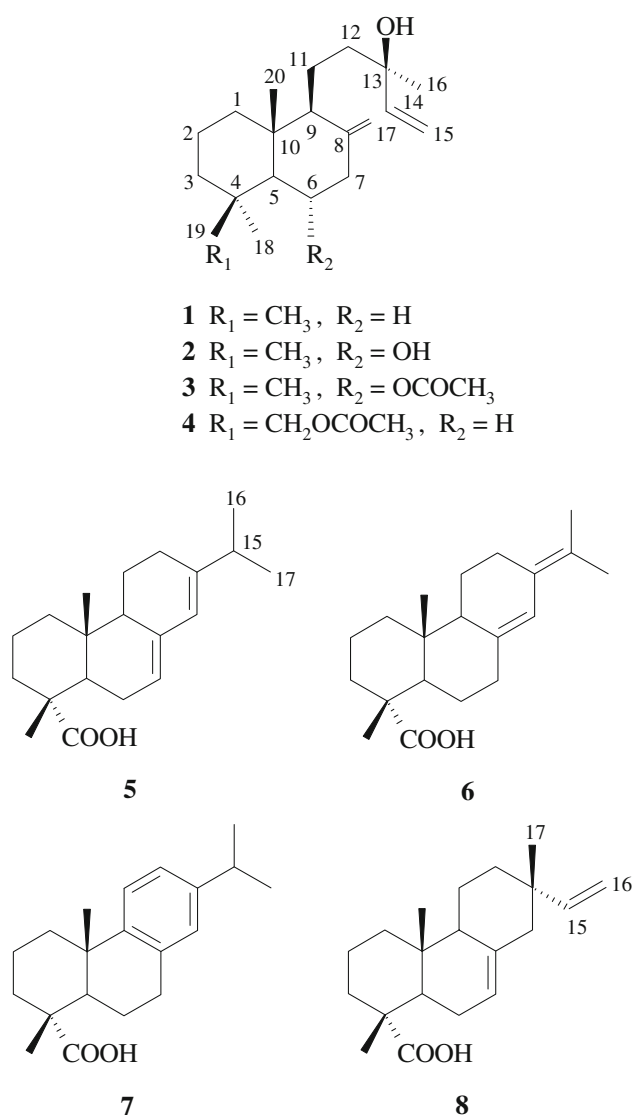
The Wilcoxon signed rank test ( $n = 12$ ) was used to compare the contents of the petroleum ether–diethyl ether extract and the diterpenoids between the rhytidome and secondary phloem. All data analysis was performed with statistical software JMP 9.0.0 (SAS Institute, Cary, NC, USA).

## Results and discussion

#### Identification of diterpenoids from the bark

The schema for separation of the diterpenoids from the diethyl ether extract of the branch bark of *L. gmelinii* var. *japonica* is shown in Fig. 1, and the isolated compounds are indicated by boldface numbers (1–8). The eight major diterpenoids isolated from the extract were identified by NMR, MS, and IR analyses. There were four labdane (1–4)-, three abietane (5–7)-, and one pimarane (8)-type diterpenoids (Fig. 2).

Compounds 1 and 2 from the neutral fraction were identified as 13-epimanool (1) [25–30] and larixol (2) [25, 27–30], respectively, by  $^1\text{H}$  NMR, MS, and IR analyses



**Fig. 2** Labdane (1–4)-, abietane (5–7)-, and pimarane (8)-type diterpenoids isolated from the diethyl ether extract of the branch bark of *Larix gmelinii* var. *japonica*

(Fig. 2). The  $^1\text{H}$  NMR spectral data of compound **3** were similar to those of larixol (**2**), except for signals indicating the presence of a  $6\alpha$ -acetoxyl group in the molecule [25]. From this information, together with the  $^{13}\text{C}$  NMR and MS spectral data, compound **3** was identified as larixyl acetate (**3**) (Fig. 2). The  $^1\text{H}$ ,  $^{13}\text{C}$  NMR, and EIMS spectral data of compound **4** were closely similar to those of torulosyl acetate in the previous literature [31, 32], except for its 17-H signal slightly shifted downfield compared with that of torulosyl acetate because of the attachment of a  $13\beta$ -hydroxy group in the molecule [25, 26]. Therefore, compound **4** was identified as 13-epitorulosyl acetate (**4**) (Fig. 2). The acidic compounds **5–8** were identified as abietic acid (**5**) [33–35], neoabietic acid (**6**) [34, 35], dehydroabietic acid (**7**) [33–35], and isopimaric acid (**8**) [36, 37] (Fig. 2), respectively, by comparing the NMR, MS, and IR data with those in the literature and/or databases of organic compounds.

Mills [38] reported the presence of seven diterpenoids (**1–3** and **5–8**) among the oleoresins of Dahurian larch (*L. gmelinii* var. *gmelinii*), which is a typical variety of *L. gmelinii* that widely occurs in east Siberia, northeast Mongolia, and northeast China. In addition, Chernenko et al. [39] showed the presence of eight diterpenoids (**1–8**), including compound **4**, in the petroleum ether extract of the bark of *L. gmelinii* var. *gmelinii*. Thus, the major diterpenoids biosynthesized in *L. gmelinii* var. *japonica* were similar to those biosynthesized in *L. gmelinii* var. *gmelinii*.

Constitutive diterpenoids in the rhytidome and secondary phloem

#### Quantitative composition of constitutive diterpenoids in both bark tissues

Table 1 shows the contents of the petroleum ether–diethyl ether extract (1:1 v/v) and the composition of the major constitutive diterpenoids in the rhytidome and secondary phloem in the branch bark of *L. gmelinii* var. *japonica*. The ether extract content in the rhytidome was much higher than that in the secondary phloem ( $P < 0.001$ , Table 1). All eight diterpenoids, i.e., 13-epimanool (**1**), larixol (**2**), larixyl acetate (**3**), 13-epitorulosyl acetate (**4**), abietic acid (**5**), neoabietic acid (**6**), dehydroabietic acid (**7**), and isopimaric acid (**8**), were confirmed in both types of bark tissues (rhytidome and secondary phloem). However, the amounts of these diterpenoids were significantly higher in the rhytidome than in the secondary phloem ( $P = 0.001$  and  $P < 0.001$ , respectively, Table 1).

As noted in a previous report [15], the raw fat (diethyl ether extract) content including oleoresins was higher in the rhytidome than in the secondary phloem in the 36- to 42-year-old stems of *L. gmelinii* var. *japonica*, *L. kaempferi*, *L. decidua* (European larch), and *L. gmelinii* var. *olgensis* (Korean larch). In *Morus alba* (white mulberry) of woody angiosperms, the contents of volatile oils and resins (dichloromethane extract) containing terpenoids were higher in the rhytidome than in the secondary phloem [40].

**Table 1** Contents of the petroleum ether–diethyl ether extract and composition of the constitutive diterpenoids in the rhytidome and secondary phloem in the branch bark of *Larix gmelinii* var. *japonica*

	Rhytidome (mg/g) <sup>a</sup>			Secondary phloem (mg/g) <sup>b</sup>			<i>P</i> <sup>c</sup>
	Mean	Range	SD	Mean	Range	SD	
Petroleum ether–diethyl ether extract	62.31	45.56–84.52	14.03	48.70	31.62–74.63	13.69	<0.001
Diterpenoids							
13-Epimanool ( <b>1</b> )	1.97	0.71–3.47	0.96	1.52	0.34–3.09	0.90	<0.001
Larixol ( <b>2</b> )	3.84	2.09–6.25	1.27	2.47	1.26–3.47	0.75	<0.001
Larixyl acetate ( <b>3</b> )	2.28	0.40–4.00	1.09	1.51	0.34–3.22	0.76	<0.001
13-Epitorulosyl acetate ( <b>4</b> )	1.87	0.96–3.24	0.66	1.20	0.49–2.25	0.57	<0.001
Abietic acid ( <b>5</b> )	1.86	0.91–3.09	0.67	1.07	0.40–1.95	0.43	<0.001
Neoabietic acid ( <b>6</b> )	0.27	0.12–0.44	0.11	0.24	0.11–0.41	0.11	0.001
Dehydroabietic acid ( <b>7</b> )	0.60	0.32–1.03	0.26	0.39	0.20–0.64	0.13	0.001
Isopimaric acid ( <b>8</b> )	2.17	1.44–3.31	0.59	1.37	0.62–2.84	0.65	<0.001
Total of the diterpenoids	14.60	8.24–19.78	3.59	9.53	6.00–11.88	1.99	<0.001

*n* = 12

<sup>a</sup> Based on the dry weight of the rhytidome

<sup>b</sup> Based on the dry weight of the secondary phloem

<sup>c</sup> *P* values resulted from analysis of the differences in the ether extract and diterpenoid contents between the rhytidome and secondary phloem in the branch bark of each individual tree using the Wilcoxon signed rank test

From the viewpoint of bark chemistry and anatomy, Srivastava [41] pointed out that the concentrations of fatty materials, such as waxes, fats and fatty acids, volatile oils, resins, and higher alcohols, in extractive fractions from the periderm and rhytidome were higher than those from the secondary phloem. These findings were analogous to the above results that showed the maldistribution of the constitutive diterpenoids in the rhytidome of *L. gmelinii* var. *japonica* (Table 1).

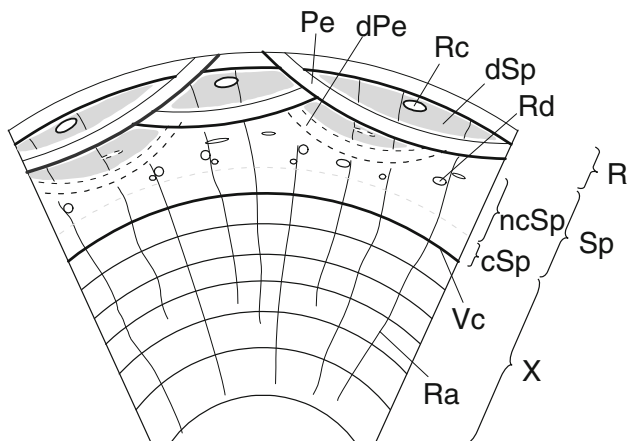
#### Accumulation mechanisms of constitutive diterpenoids in the bark tissues

Figure 3 shows a transverse section image of the tissues in the *Larix gmelinii* var. *japonica* branch with a well-developed rhytidome. In living secondary phloem of conifers, especially Pinaceae trees, mono-, sesqui-, and diterpenoids are biosynthesized in epithelial cells within internal (endogenous) secretory structures, i.e., resin cysts, ducts, canals, and cavities [21, 23, 42]. These terpenoids are transferred to and stored in the lumen of these structures as oleoresins [21, 23]. From the viewpoint of physiological function of tissue, the secondary phloem is further separated into the inner and outer part, i.e., the conducting and non-conducting phloem, respectively, in Fig. 3 [20, 21, 41, 43, 44]. The non-conducting phloem accumulates various secondary metabolites, such as phenolic and resinous substances [2, 21, 41, 43]. It was usually observed that horizontal resin ducts and parenchyma cells became

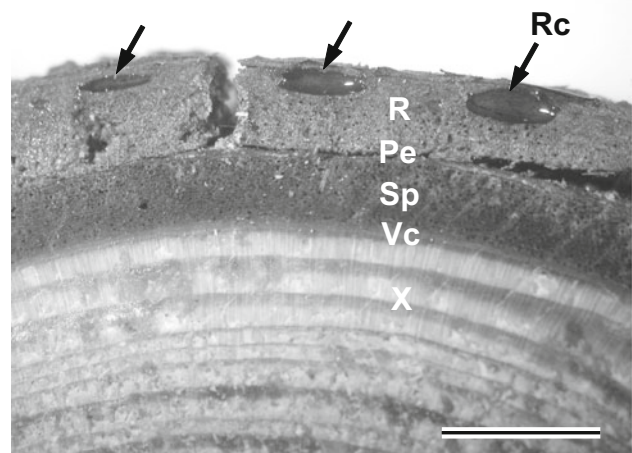
conspicuously enlarged in the non-conducting phloem in many *Larix*, *Pinus*, and *Picea* species of Pinaceae, including *L. occidentalis* (Western larch) and *L. laricina* (American larch) [43–46], due to the tangential stresses by secondary dilatation growth [21, 41]. Consequently, intracellular space volume for storage of oleoresins containing terpenoids may be larger in the non-conducting phloem than in the conducting phloem, in the larch species including *L. gmelinii* var. *japonica*.

Rhytidome is composed of dead and dying layers (Fig. 3). The layers are formed by the successive development of the periderm and its separation from the outer part of the living secondary phloem, i.e., the non-conducting phloem [2, 20, 21]. Then, the secondary phloem captured into the rhytidome becomes dead tissue [2, 21, 44] and would continue to store oleoresins. In this process, the constitutive diterpenoids (1–8) would be accumulated in the rhytidome of *L. gmelinii* var. *japonica* (Table 1).

An optical microscopy image of a transverse section of *L. gmelinii* var. *japonica* branch is shown in Fig. 4. Some developed fusiform resin cavities filled with oleoresins were usually observed in the dead secondary phloem within the rhytidome. A previous study [15] also reported that fusiform resin cysts or cavities including oleoresins were normally located in the bark tissues of 7-year-old branch of *L. gmelinii* var. *japonica*. Therefore, the diterpenoid maldistribution in the rhytidome of *L. gmelinii* var. *japonica* branch (Table 1) may be partly attributable to the



**Fig. 3** Transverse section diagram of the tissues in the *Larix gmelinii* var. *japonica* branch with well-developed rhytidome. Living secondary phloem (Sp) is composed of inner and outer part, i.e., conducting phloem (cSp) and non-conducting phloem (ncSp), respectively. Rhytidome (R) consists of dead and dying layers. The layers are constituted by the consecutive development of the periderm (Pe) and the separation from the non-conducting phloem. Other details: X secondary xylem, Vc vascular cambium, dSp dead secondary phloem, dPe developing periderm, Rc resin cavity, Rd resin duct, Ra ray



**Fig. 4** Transverse section of a partial circumference of a *Larix gmelinii* var. *japonica* branch, which includes the rhytidome (R), periderm (Pe), secondary phloem (Sp), vascular cambium (Vc), and secondary xylem (X). There are some developed fusiform resin cavities (Rc; arrows) containing oleoresin in the rhytidome. The fusiform shape of the resin cavities and the central crack on this part owing to the secondary dilatation growth of the branch. Scale bar 5 mm

usual existence of such enlarged resin cavities full of oleoresins in the rhytidome (Fig. 4).

### Implications for ecological aspects

Gershenzon and coworkers [47, 48] suggested that in the natural world, high concentrations of defensive substances on the outer parts of plant bodies, such as terpenoids in glandular trichomes, could present a very effective barrier against herbivores. Similarly, the maldistribution of constitutive diterpenoids in the outermost tree tissue (rhytidome) could also be a highly effective defense against natural enemies for preventing significant damage.

Terpenoids are potentially toxic to living plant tissues; hence, terpenoid storage might serve to limit the risk of toxicity to the plant itself [47]. In addition, some types of terpenoids, especially resin acids with non-aromatic conjugated dienes, are easily oxidized by air and/or sunlight [49]. Thus, diterpenoid accumulation in the rhytidome, which is dead tree tissue shelled with a non-permeable suberized cork layer, would be prevented from harming other living tissues in the tree's own body and also be protected from chemical deterioration.

### Conclusions

The major constitutive diterpenoids in the branch bark tissue of *L. gmelinii* var. *japonica* were isolated and identified as 13-epimanol (1), larixol (2), larixyl acetate (3), 13-epitorulosyl acetate (4), abietic acid (5), neoabietic acid (6), dehydroabietic acid (7), and isopimaric acid (8). These diterpenoids were present in both bark tissues, the rhytidome and secondary phloem. However, the concentrations of these individual diterpenoids were higher in the rhytidome than in the secondary phloem. Enlarged resin cavities filled with oleoresins were usually found in dead secondary phloem embedded in the rhytidome. The accumulation and distribution of these diterpenoids in the bark tissues are probably caused by terpenoid biosynthesis in the living secondary phloem and the process of rhytidome formation. In terms of constitutive chemical defense of conifers, our findings show that the rhytidome provides safe and stable storage of the potentially self-toxic diterpenoids at high concentrations that may provide an effective defense against natural enemies.

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### References

- Laver ML (1991) Bark. In: Lewin M, Goldstein IS (eds) Wood structure and composition. Marcel Dekker, New York, pp 409–434
- Kučera LJ, Bergamin L (2003) The structure, function and physical properties of bark. In: Vaucher E, Eckenwalder JE (eds) Tree bark: a color guide. Timber Press, Portland, pp 23–37
- Lewinsohn E, Gijzen M, Croteau R (1991) Defense mechanisms of conifers: differences in constitutive and wound-induced monoterpene biosynthesis among species. *Plant Physiol* 96:44–49
- Phillips MA, Croteau R (1999) Resin-based defenses in conifers. *Trends Plant Sci* 4:184–190
- Franceschi VR, Krokene P, Christiansen E, Krekling E (2005) Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol* 167:353–376
- Raffa KF, Berryman AA (1982) Accumulation of monoterpenes and associated volatiles following inoculation of grand fir with a fungus transmitted by the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae). *Can Entomol* 114:797–810
- Langenheim JH (1994) Higher plant terpenoids: a phytocentric overview of their ecological roles. *J Chem Ecol* 20:1223–1280
- Yamada T (2001) Defense mechanisms in the sapwood of living trees. *J For Res* 6:127–137
- Keeling CI, Bohlmann J (2006) Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defense of conifers against insects and pathogens. *New Phytol* 170:657–675
- Keeling CI, Bohlmann J (2006) Diterpene resin acids in conifers. *Phytochemistry* 67:2415–2423
- Inukai T, Haga R (1953) Experimental studies on the taste of the vole for various kinds of the larch (in Japanese). *Mem Fac Agric Hokkaido Univ* 1:281–300
- Takahashi N, Nishiguchi C (1966) Studies on the resistance of forest trees to red-backed vole, *Clethrionomys rufocanus bedfordiae* (Thomas), I: relative feeding preference of the vole for seedlings of conifers under laboratory conditions (in Japanese). *Bull Tokyo Univ For* 62:153–172
- Chiba S, Nagata Y (1976) Studies on the breeding of *Larix* species, V: experimental estimation of the vole resistance and heritability among hybrids, back-crosses, and triple hybrids (in Japanese). *Bull Oji Inst For Tree Improv* 3:33–44
- Ishihara K, Matsukawa A (1941) Forest tree breeding and improvement, II: traits of F<sub>1</sub> hybrids of conifer trees (in Japanese). In: Proceedings of the S15 Annual Meeting of Japan Forest Society, pp 126–131
- Yanagisawa S, Kawanishi T (1955) On the rinds of four species of the *Larix* genus (in Japanese). *Bull Gov For Exp Stn* 79:125–142
- Nishiguchi C, Arisawa H, Iizuka T (1977) Studies on the resistance of forest trees to the red-backed vole, *Clethrionomys rufocanus bedfordiae*, IV: chemical substances taking part in the resistance of Sakhalin larch, *Larix gmelinii* to biting by the vole (in Japanese). *J Jap For Soc* 59:167–172
- Hayashi E, Iizuka K, Sukeno S, Kohno K (1998) Relationship between resistance to vole browsing and content of ether extract in bark of larch species and hybrids. *J For Res* 3:119–122
- Sukeno S, Ozawa S (1997) Identification of compounds found in the larch associated with resistance to vole, and its application for

- early test, III: identification of compounds associated with vole resistance (in Japanese). Abstracts of the 108th Annual Meeting of Japan Forest Society, pp 198
19. Sato M, Seki K, Kita K, Moriguchi Y, Hashimoto M, Yunoki K, Kofujita H, Onishi M (2009) Comparative analysis of diterpene composition in the bark of the hybrid larch *F<sub>1</sub>*, *Larix gmelinii* var. *japonica* × *L. kaempferi* and their parent trees. *J Wood Sci* 55:32–40
  20. Trockenbrodt M (1990) Survey and discussion of the terminology used in bark anatomy. *IAWA Bull* ns 11:141–166
  21. Evert RF (2006) *Esau's plant anatomy: meristems, cells, and tissues of the plant body; their structure, function, and development*, 3rd edn. Wiley, Hoboken
  22. Kozłowski TT, Pallardy SG (1997) *Physiology of woody plants*, 2nd edn. Academic Press, San Diego
  23. Langenheim JH (2003) *Plant resins: chemistry, evolution, ecology, and ethnobotany*. Timber Press, Portland
  24. Hansson L, Gref R, Lundren L, Theander O (1986) Susceptibility to vole attacks due to bark phenols and terpenes in *Pinus contorta* provenances introduced into Sweden. *J Chem Ecol* 12:1569–1578
  25. Norin T, Ohloff G, Willihalm B (1965) The structures and configurations of larixol and larixyl acetate. *Tetrahedron Lett* 6:3523–3528
  26. Bruns K (1970) Absolute configuration at position 13-C atom in labdane-type diterpenoids (in German). *Tetrahedron Lett* 11:3263–3264
  27. Manning TR (1973) Diterpene constituents of *Pinus contorta* bark. *Aust J Chem* 26:2735–2739
  28. Fang JM, Wu SK, Chen YS (1985) A study of the constituents of the bark of *Tsuga chinensis* Pritz. var. *formosana* (Hay.). *J Chin Chem Soc* 32:477–480
  29. Dev Sukh, Misra R (1985) *Handbook of terpenoids: diterpenoids, II: bicyclic diterpenoids*. CRC Press, Boca Raton
  30. Enzell CR, Ryhage R (1965) Mass spectrometric studies of diterpenes, I: carbobicyclic diterpenes. *Arkiv Kemi* 23:367–399
  31. Piovetti L, Gonzale E, Diara A (1980) Diterpene composition of *Cupressus dupreziana* and *Cupressus sempervirens*. *Phytochemistry* 19:2772–2773
  32. Hanari N, Ono Y, Ooi T, Yamamoto H (1998) Diterpenes extracted by a polar solvent from the bark of the resinous stem canker of *Chamaecyparis obtusa* (in Japanese). *Mokuzai Gakkaishi* 44:342–350
  33. National Institute of Advanced Industrial Science and Technology, AIST (2004) SDBS Web. <http://riodb01.ibase.aist.go.jp/sdbs/>. Accessed 14 Sep 2010
  34. National Institute of Standards and Technology, NIST (2008) NIST Chemistry Web Book. <http://webbook.nist.gov/chemistry/>. Accessed 14 Sep 2010
  35. Sadhra S, Gray CN, Foulds IS (1997) High-performance liquid chromatography of unmodified rosin and its applications in contact dermatology. *J Chrom B* 700:101–110
  36. Wenkert E, Afongo A, Beak P, Carney RWJ, Jeffs PW, McChesney JD (1965) The proton magnetic resonance spectral characteristics of tricyclic diterpenic substances. *J Org Chem* 30:713–722
  37. Kuo YH, Chen WC (1999) Chemical constituents of the pericarp of *Platycladus orientalis*. *J Chin Chem Soc* 46:819–824
  38. Mills JS (1973) Diterpens of *Larix* oleoresins. *Phytochemistry* 12:2407–2412
  39. Chernenko GF, Ivanova EE, Demenkova LI, Shmidt ÉN (1991) Extractive substances in the bark of *Larix dahurica* (in Russian). *Khim Priir Soedin* 4:580–582
  40. Ishii Y, Ohara S (2004) Quantitative difference of the components between the inner bark and the outer bark of *Morus alba* L (in Japanese). *J Jpn For Soc* 86:372–374
  41. Srivastava LM (1964) Anatomy, chemistry, and physiology of bark. In: Romberger JA, Mikola P (eds) *International review of forestry research*. I. Academic Press, San Diego, pp 203–277
  42. Croteau R, Johnson MA (1985) Biosynthesis of terpenoid wood extractives. In: Higuchi T (ed) *Biosynthesis and biodegradation of wood components*. Academic Press, Orlando, pp 379–439
  43. Srivastava LM (1963) Secondary phloem in the Pinaceae. *Univ Calif Publ Bot* 36:1–141
  44. Chang YP (1954) *Anatomy of common North American pulpwood barks*. TAPPI monograph series No. 14. TAPPI, New York
  45. Wu H, Hu ZH (1997) Comparative anatomy of resin ducts of the Pinaceae. *Trees* 11:135–143
  46. Chang YP (1954) *Bark structure of North American conifers*. Technical Bulletin No. 1095. USDA Forest Products Laboratory, Madison
  47. Gershenzon J (1994) Metabolic costs of terpenoid accumulation in higher plants. *J Chem Ecol* 20:1281–1328
  48. Wittstock U, Gershenzon J (2002) Constitutive plant toxins and their role in defense against herbivores and pathogens. *Curr Opin Plant Biol* 5:300–307
  49. Enoki A, Kitao K (1975) Autoxidation of resin acids, III: photoxidation of abietic acid. *Mokuzai Gakkaishi* 21:101–106